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FIELD EMISSION SCANNING ELECTRON MICROSCOPY OF THE INTERACTION OF CLOSTRIDIUM DIFFICILE WITH HUMAN EMBRYONIC INTESTINAL CELLS

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An in vitro assay system, consisting of monolayers of human embryonic intestinal cells (HEI) and Clostridium difficile (C. difficile), was used to observe interactions of microorganisms with the cell surface and cytoplasm. Tests were performed using Toxin B positive C. difficile Strain 938, and Toxin B negative Strain 789, harvested from casamino acids agar (CAS), and CAS containing subinhibitory concentrations of clindamycin (CAS-CL). Adherence to the surface and internalization into HEI cells was quantified at 30, 60, and 90 min. Cryopreserved and freeze fractured preparations were made at 60 min and observed for adherence and internalization using field emission scanning electron microscopy. Quantitative microorganism determinations, confirmed by the observed microoganism activity harvested from CAS and CAS-CL, revealed their capability to adhere to and/or enter HEI ceils. The Toxin B producer, Strain 938, harvested from CAS-CL entered the cytoplasm and remained intact within it.

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PREFACE

The work described in this report was authorized under Project No. 1FJ1-2-R, Biotechnology. This work was started in January 1984* and completed in January 1991.

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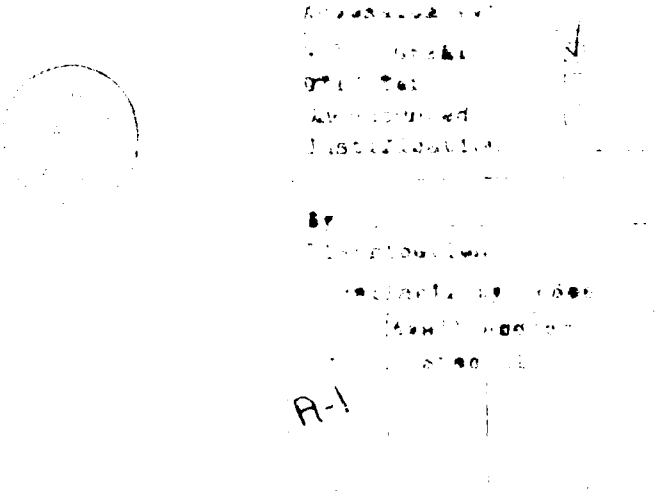

*This work started when the primary author was a student at the Medical College of Virginia, Richmond, VA.

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FIELD EMISSION SCANNING ELECTRON MICROSCOPY OF THE INTERACTION OF *CLOSTRIDIUM DIFFICILE* WITH HUMAN EMBRYONIC INTESTINAL CELLS

1. INTRODUCTION

Clostridium difficile (*C. difficile*), the etiologic agent of pseudomembranous colitis, unpredictably colonizes the bowels of certain hospitalized patients following the administration of antibiotics or during the course of a hospital stay.[1] Enterotoxins produced by this microorganism are capable of damaging the bowel mucosa;[2] but until now, it has not been determined whether [as with microorganisms such as *Escherichia coli* (*E. coli*)] adhesive factors help this microorganism colonize and proliferate before toxin production.[3-7]

Previous studies using the hamster model clarified some assumptions about proper conditions for colonization. In the hamster, diet, colonization, and competition for colonization sites, were shown to affect the outcome of an experimentally induced infection. More specifically, administration of a high percentage of protein in the diet (or total food deprivation for several days) hampered the animal's ability to resist establishment of *C. difficile* cecitis in the presence or absence of antibiotics.[8] Also, when antibiotics were administered during the course of a high percentage protein diet, experimental lifespans were shortened compared to the administration of antibiotics or increased protein alone. Evidence suggests a synergistic action of diet with antibiotics.[4,8]

Indirectly, a colonization mechanism has been elucidated in the following experiments. The presence of a nonpathogenic strain of *C. difficile* in the bowel, before challenge with a pathogenic strain, protected the bowel from colonization.[1,2,9,10] This protection was shown not to be immunologic but resulted from exclusion of the pathogenic strain from the mucosa.[11] Although increased survival time of cefoxitin-treated hamsters was observed when prior treatment with a noncytotoxigenic strain was employed, no protection was seen when cytotoxigenic and noncytotoxigenic strains were administered simultaneously. These observations show that attachment sites were blocked by nonpathogenic variants.[8] Colonization appears to be site dependent and may be influenced by the selective production of cell wall components resulting from microbial consumption of available nutrients.[5,12]

This in vitro study notes the effects of a high percentage of protein in the growth media and exposure to clindamycin on the behavior of *C. difficile* on human embryonic intestinal (HEI) cells. The role of selected nutrients and clindamycin in the predisposition of *C. difficile* to attach to cell surfaces, enter, and survive within HEI cells is shown. A combination of selected nutrients and the presence of clindamycin, in addition to the microenvironment, may be critical to the development of pseudomembranous colitis. As no valid explanation for relapse has yet been postulated, one explanation that must now be considered is survival within nonphagocytic cells in response to selected microbial nutrients and clindamycin.

2. MATERIALS AND METHODS

2.1 Organisms Used.

Clostridium difficile strains, isolated from the patient population at the Medical College of Virginia, Virginia Commonwealth University (Richmond, VA), include *C. difficile* Strain 938 from a symptomatic adult and *C. difficile* Strain 789 isolated from an asymptomatic child. Assays for the production of Toxin B[7] showed that Strain 938 caused rounding of HEI cells in 24 hr, and Strain 789 did not cause rounding after 48 hr. Rounding was inhibited by *C. difficile* antitoxin obtained from Dr. Tracey Wilkins, Virginia Polytechnic Institute and State University (Blacksburg, VA).

Identification was based on the appearance of the colony, fluorescence under a 366-nm lamp, gram stain morphology, the absence of lipase or lecithinase, and gas-liquid chromatography profiles. The *C. difficile* isolates had minimum inhibitory concentrations of 1.0 µg/mL clindamycin when tested by agar dilution susceptibility.[13]

2.2 Organism Preparation for Adherence Assay.

Microorganisms were grown on casamino acids agar (CAS) consisting of 1% casamino acids (DIFCO Labs, Detroit MI), 2% agar (DIFCO), and 0.5% sheep blood (Flow Labs, McLean, VA). To expose the organisms to subinhibitory concentrations of clindamycin, 0.05 µg/mL clindamycin was added to the CAS media. Organisms were harvested at 48 hr in Eagle's minimal essential media (MEM) (Flow Labs) containing 1% fetal calf serum (FCS) (GIBCO, Grand Island, NY), pH 5.5, washed once, and resuspended in MEM 1% FCS. Antitoxin at a 1:10 dilution in 0.05 mL MEM was used in an attempt to block adherence. This mixture was incubated at 37 °C in an anaerobe jar with the atmosphere generated by an anaerobe gas pack [Becton Dickinson Microbiology Systems (BDMS), Cockeysville, MD] for 15 min. The preparations were centrifuged at 1500 rpm for 5 min and resuspended in MEM with 1% FCS, pH 5.5.

2.3 Tissue Cells Used.

Human embryonic intestinal cells #CCL6 were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in HAM's F12 medium (GIBCO) containing 10% FCS (GIBCO) and penicillin/streptomycin (DIFCO), 50 units and 50 µg/mL, respectively.

2.4 Assay for Cytotoxigenicity.

Procedures for the determination of cytotoxigenicity were followed as previously described.[7] Briefly, the cytotoxin assay tested supernatent dilutions from each microorganism for rounding of HEI cells from 4 to 48 hr after inoculation. If rounding was observed, and this rounding was blocked using antitoxin to Toxin B, then the organisms were considered cytotoxigenic.

2.5 Adherence Assay.

The adherence assay was performed using C. difficile grown on CAS as previously described. Earlier studies measured adherence of seven different isolates under five different conditions, in duplicate.[7] Briefly, microorganisms were inoculated onto cell monolayers, incubated anaerobically on a rotating platform for 15 min at 37 °C, washed, stained with Giemsa stain, and read. The percentage of cells (number of cells out of 100 with adherent microorganisms) with microorganisms adhering was calculated. Areas with low background adherence were chosen for these counts.

To determine whether adherence depended on microbe viability, viable and nonviable microorganisms were used initially. Viable C. difficile were taken from CAS plates, which had been incubated anaerobically for 48 hr. Nonviable microorganisms were taken from blood agar plates, which had been stored aerobically for 3 days. Subcultures from aerobic plates failed to produce growth when plated on sheep blood agar (SBA) and incubated anaerobically at 37 °C for 48 hr. Gram stains confirmed intact organisms.

2.6 Internalization Assay.

Adherence assay procedures were followed with several exceptions: assays were carried out in 24-well Falcon plates (BDMS); confluent monolayers were inoculated with approximately 5 x 10^5 organisms; five wells per test condition were harvested; and incubation was in gas pak pouches (BDMS) generating an anaerobic environment. At 30, 60, and 90 min, monolayers were washed to remove loose microorganisms, and a 1% solution of Triton X100 was placed on the monolayer and incubated at room temperature for 5 min. After 5 min, the microorganisms were harvested, dilutions were made, and microorganisms were subcultured to SBA and incubated anaerobically for 48 hr at which time colony counts were established. To the same 24-well tissue culture plate, in different test wells, clindamycin at 10 µg/mL was added to kill any microorganisms attached to the monolayer surface. After incubation in an anaerobe pouch for 90 min, these test wells were washed to remove dead organisms, and a 1% solution of Triton X100 was added for 5 min. The microorganisms were harvested, diluted, and plated on SBA for subsequent colony counts. To determine the percentage of internalized microorganisms, a ratio of internalized organisms to total cell associated organisms x 100 was obtained. Microorganisms 789 and 938, harvested from CAS and CAS containing subinhibitory concentrations of clindamycin (CAS-CL), were used.

2.7 Field Emission Scanning Electron Microscopy, Cryopreservation, and Freeze Fracture.

The adherence assay was performed as previously described.[7] Preparations were rinsed in buffer and fixed in 3% gluteraldehyde at room temperature after a 60-min incubation at 37 °C. Cryopreservation (Strain 938, CAS) in liquid nitrogen was performed, and etching was used to achieve clarity of the image. Freeze fracture (Strain 938, CAS-CL) was performed by freezing the coverslip containing the cell/microorganism layer in liquid nitrogen, then manually fracturing the coverslip inside a cold chamber.

3. RESULTS

3.1 Cytotoxigenicity Assay.

The in vitro assay for the presence of Toxin B showed rounding of 100% HEI cells at 24 hr by Strain 938. Strain 789, however, caused no rounding of HEI cells after 48 hr.

3.2 Adherence Assay.

Adherence assays previously performed, in duplicate, on seven different isolates gave a 60% average of cells with microorganisms attached. The preparative adherence assay performed prior to this set of experiments showed 62 and 67% attachment for Strains 938 and 789, respectively. In preliminary experiments comparing attachment of viable microorganisms to attachment of nonviable microorganisms, only viable microorganisms attached, which indicated that an active process was at work in the attachment phenomena.

3.3 Internalization Assay.

As seen in the table and Figure 1, the ratio of internal to total cell associated organisms was highest with Strain 938 harvested from CAS-CL at 60 min. Data from the in vitro adherence assay was substantiated by reproducibility of the in vitro results (data not shown) and electron micrograph results.

3.4 Field Emission Scanning Electron Microscopy.

In Figure 2, the cryopreserved preparation of Strain 938 harvested from CAS, showed intimate interaction with HEl cells. In Figure 3, Strain 938 harvested from CAS-CL, shown by freeze fracture, is intact within HEI cells. (Microorganisms are shown with arrows.)

4. DISCUSSION

Adults develop C. difficile associated colitis following the administration of antibiotics; whereas, infants harbor C. difficile in the bowel but rarely show signs of disease. In addition, historical data has shown that intravenous and intramuscular administration of antibiotics are associated with the development of pseudomembranous colitis; but, a direct correlation of disease symptoms to dose does not exist.[14] Predisposing environmental conditions in the bowel, which are indirectly related to antibiotic administrations, have been implicated in the development of pseudomembranous colitis. These conditions include disruptions in the protective layer of the bowel, selected antibiotic administration, changes in health or nutritional status of the host, food deprivation, and a designated diet or change in diet.[10] Changes in the organism instead of in the environmental conditions in the bowel have not yet been considered. Clostridium difficile strains differ in their ability to produce cytotoxin, in the presence of cell wall components such as adhesins, in their response to the immediate environment, in the use of selected nutrients, and in their proximity to the bowel mucosa. Organism changes in response to growth conditions cannot be discounted as contributive to the development of pseudomembranes.

TABLE. Numbers of Total Cell Associated C. difficile and Internalized C. difficile. Percentages were obtained by dividing the number of internalized organisms by the number of total cell associated organisms and multiplying by 100.

Organism/ conditions	Total cell-associated organisms CFU/mL	Internalized organisms CFU/mL	Percentage internal/ total x 100
938 CAS			
30 min	5.20×10^4	1.30×10^4	25.0
60 min	5.80×10^4	1.74×10^4	30.0
90 min	1.55×10^5	4.50×10^3	2.9
938 CAS-CL			
30 min	4.00×10^4	1.20×10^4	30.0
60 min	3.50×10^4	4.40×10^4	125.7
90 min	1.00×10^6	4.75×10^3	0.5
789 CAS			
30 min	1.40×10^4	4.80×10^2	3.4
60 min	5.00×10^3	1.75×10^3	35.0
90 min	2.85×10^3	1.30×10^2	4.5
789 CAS-CL			
30 min	2.00×10^4	8.00×10^2	4.0
60 min	7.20×10^3	1.78×10^3	24.7
90 min	1.75×10^4	4.50×10^2	2.6

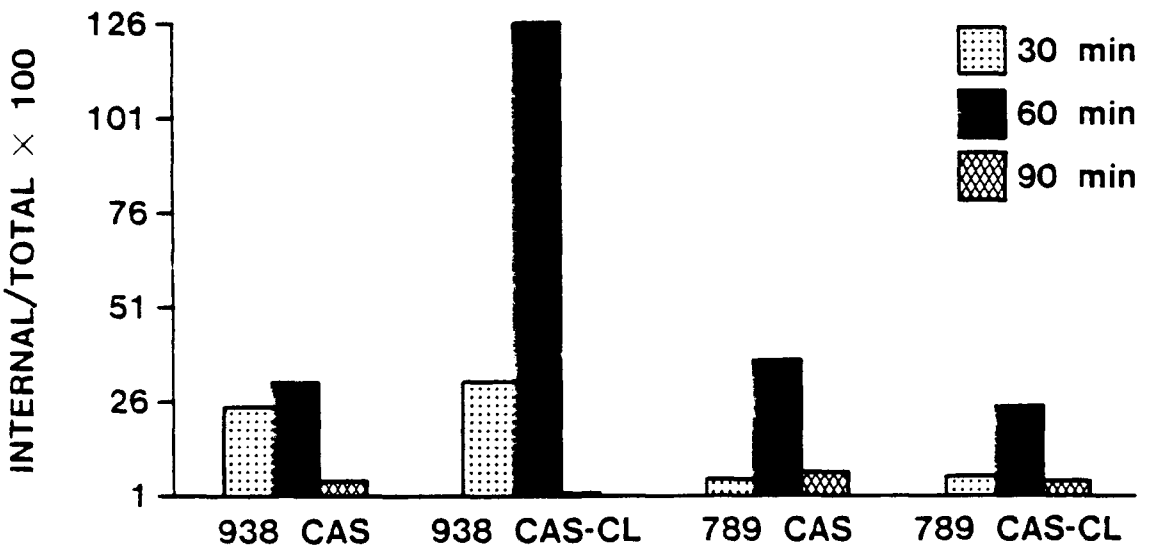

Figure 1. Internalization of C. difficile by Human Embryonic Intestinal Cells. Ratio of internalized microorganisms versus total cell associated microorganisms at 30, 60, and 90 min. The greatest ratio was observed with C. difficile Strain 938 harvested from CAS-CL.

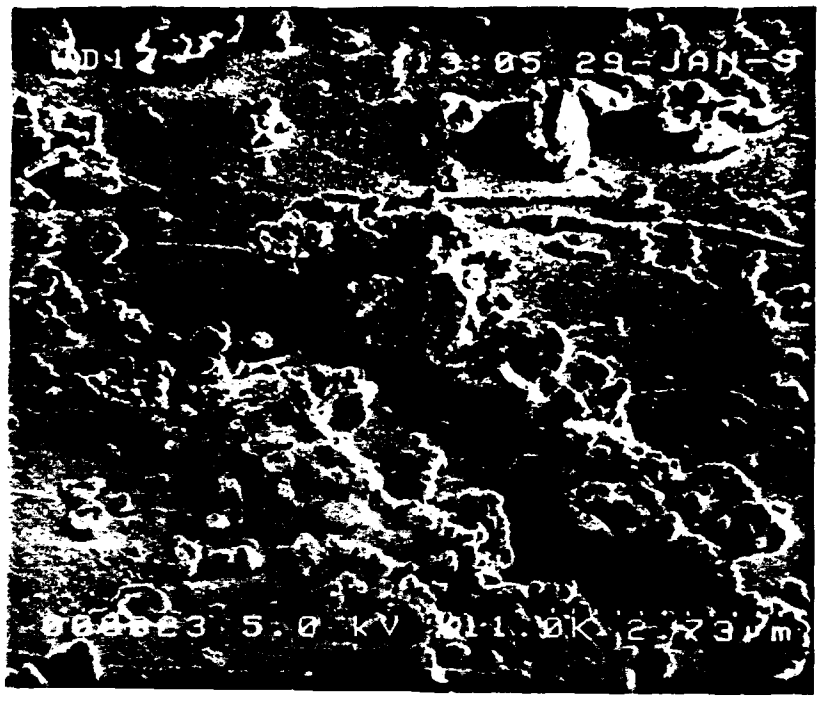

Figure 2. Field Emission Scanning Electron Microscopy of C. difficile Strain 938, Harvested from CAS. Intimate association with the cells is apparent. Interaction was stopped by fixation at 60 min, and the sample was cryopreserved prior to observation. Bar = 2.73 μ.

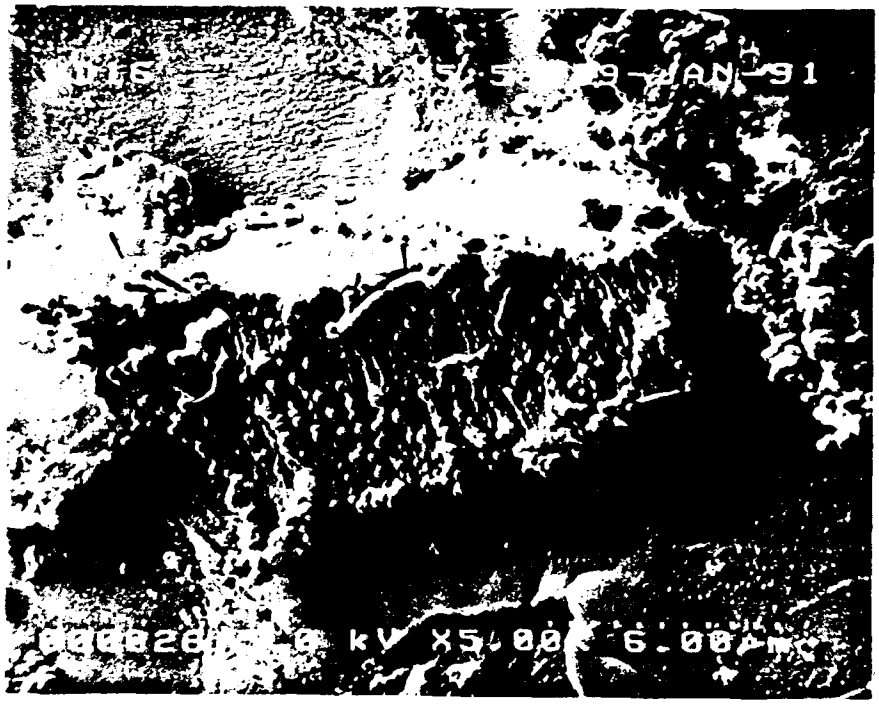

Figure 3. Field Emission Scanning Electron Micrograph of a Freeze Fractured Preparation of C. difficile, Harvested from CAS Containing 0.05 μg/mL Clindamycin, Intact Within Human Embryonic Intestinal Cells After 60 min. Organisms, as shown by the arrows, are numerous and appear unharmed. Bar = 6.0 μ.

Initial results, presented here, suggest a direct effect of available nutrients (amino acids) and clindamycin on the microorganisms. Entrance into and survivability within HEI cells occurred with Toxin B producer, Strain 938, when harvested from media containing amino acids and clindamycin. Ratios of internalized microorganisms to total cell associated microorganisms were determined. Clostridium difficile Strain 938, harvested from CAS-CL were internalized and survived in high numbers at 60 min. When grown on CAS containing no clindamycin, levels of internalization and survival were not as great.

Adhesins have been implicated as an important initiating factor in colonization by enterotoxigenic E. coli.[15] In the case of C. difficile, such an adhesin may play a role in disease production by serving as the initial anchor to the intestinal epithelium. Response of the microorganism to an environmental signal and/or regulatory control and its role in disease production remain to be determined.[15-17]

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